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High Performance Liquid Chromatographic Analyses of Higenamine Enantiomers in Aconite Roots

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Abstract – The enantiomers of higenamine were directly separated by high performance liquid chromatography using a chiral stationary phase and detected by UV. The *R*- and *S*-isomers of higenamine were eluted at the retention time of 22 min and 27 min, respectively. Higenamine was determined to be present as *R*-(+)-enantiomer not only in the embryo of *Nelumbo nucifera,* from which the separation of *R*-(+)-higenamine was reported, but also in various Aconite roots, from which higenamine was separated as optically inert racemic mixtures.
Key words – Higenamine, enantiomers, *R*-(+)-higenamine, *Aconitum sp.* HPLC-chiral column

Introduction

Higenamine, 1-(*p*-hydroxybenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, was isolated as a cardiotonic component from Aconite root (Kosuge and Yokota, 1976; Kosuge *et al.*, 1978a) which has long been considered as one of the most important herbal drugs and used as a heart stimulant, diuretic and analgesic in oriental medicine (Bensky and Gamble, 1986; Tang and Eisenbrand, 1992).

Higenamine has one asymmetric carbon in the structure and exists as two enantiomers. Enantiomers usually show different degrees of activities and toxicities in biological systems and two enantiomers of higenamine also are known to show differences. *S*-(-)-higenamine was reported to have a stronger cardiotonic activity than *R*-(+)-higenamine (Kosuge and Yokota, 1976; Ho and Ko, 1985). Stadler and Zenk (1990) described that *S*-(-)-higenamine is a central intermediate in the biosynthesis of various benzylisoquinoline alkaloids in plants. Higenamine has been isolated from plants of four different genera including *Aconitum* sp., *Asiasari* sp., *Annona squamosa* and *Nelumbo nucifera* (Koshiyama *et al.*, 1970, Kosuge *et al.*, 1978a, 1978b, Leboeuf and Cave, 1981, Kosuge and Yokota, 1976). Higenamine was isolated as *R*-(+)-form from *Nelumbo nucifera* and as racemic mixtures from the other three genera.

Asymmetric synthesis of organic compounds results in a 1:1 mixture of the two enantiomeric forms, commonly known as racemates. However, most of the biosynthetic pathways are known to proceed stereo selectively yielding only one of the stereoisomers. Higenamine was also speculated to be synthesized as either one of the enantiomers in the chiral environment of plant biosynthetic systems. In this paper, the authors established an analytical method for higenamine enantiomers by using HPLC with a chiral stationary phase and the enantiomeric states of higenamine in plants were identified.

Materials and Methods

Reagents, materials and instruments – Higenamine (racemate) was synthesized as a hydrobromide salt according to the previously reported method (Chang *et al.*, 1984). Authentic samples of *S*-(-)- and *R*-(+)-higenamine are gift of Dr. Arnold Brossi of National Institutes of Health, U.S.A. Amberlite XAD-2 resin (particle size: 0.15×0.20 mm which was purchased from Serva Co., Heidelberg, F.R.G.) was activated by washing with acetone, MeOH and H_2O successively before using. HPLC-grade solvents (Fisher Scientific Co., U.S.A.) were used for the preparation of mobile phase.

Aconitum kusnezofi and *Aconitum napiforme* were collected at Mt. Seorak and Mt. Jiri in October, 1993 by Prof. Young Bae Suh, Natural Products Research Institute, Seoul National University. The Processed

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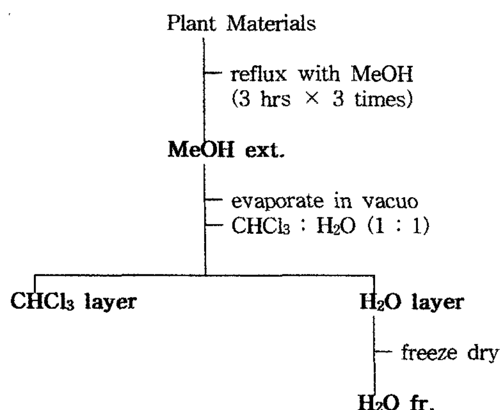
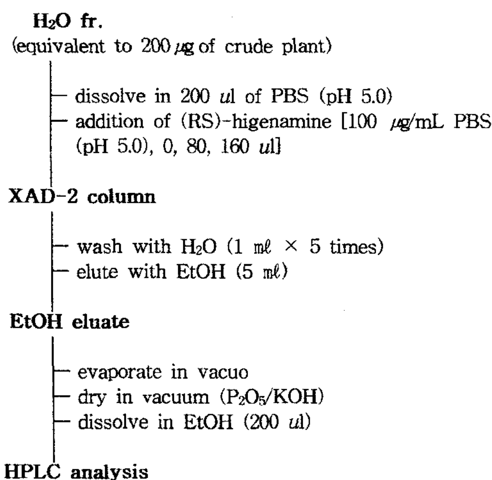
Aconite Root powder was the product of Sang Hwa Pharmaceutical Company, Japan and the other crude plant drugs were purchased from local crude drug market and were identified by Prof. Hyung Joon Chi, Natural Products Research Institute, Seoul National University, Korea.

The High Performance Liquid Chromatography System (Spectra Physics Co., U.S.A.) consisted of a Ternary Pump (SP 8800), UV Variable Wavelength Detector (SP 100), an Integrator (SP 4270), an Injector (Rheodyne 7125), and a chiral column (4.6×250 mm, CHIREX 3020 G-EO, Phenomenex Co., U.S.A.).

Extraction and pre-treatment of the plant samples – Each plant material was finely ground and extracted with MeOH with reflux. As shown in Scheme 1, the extract, after removal of the solvent, was suspended in distilled water and washed with chloroform and the H_2O layer was freeze dried. The amount of freeze dried powder equivalent to 200 μg of crude plant material was dissolved in 200 μl phosphate buffered saline (PBS, pH 5.0) and applied to a column (pasteur pipette, 0.6×2.0 cm) of pre-activated XAD-2 (2 g) and treated as shown in Scheme 2. The column was washed with distilled water (5 ml) and then eluted with 5 ml of ethanol. The eluate was concentrated and dried *in vacuo* under P_2O_5 and KOH. The residue was dissolved in 200 μl of ethanol and 5 μl each of the EtOH solution was injected into the HPLC system for analysis. For the quantitative analysis of higenamine enantiomers with the standard addition method, 80 or 160 μl of higenamine (racemate) solution (100 μg of higenamine as free base in 1 ml of PBS, pH 5.0) was applied to an XAD-2 column together with the PBS solution of the H_2O fr., treated the same as above and injected into the HPLC system.



Scheme 1. Extraction procedure of the plant sample.



Scheme 2. Pre-treatment of plant extract (H_2O fr.) for HPLC analysis.

Analysis with HPLC – A chiral column (CHIREX-3020G-EO, Phenomenex Co.) was used as a stationary phase and eluted with the mobile phase of hexane:dichloroethane:(trifluoroacetic acid/EtOH; 1/20) = 53:35:12 with a flow rate of 0.8 ml/min and the eluent was detected with UV at 284 nm.

Results and Discussion

An ordinary reversed phase column was first evaluated for the separation of the derivatized enantiomers of higenamine. (±)-Higenamine was converted to (±)-higenamine-1-phenylethylurea with *S*-(-)-1-phenylethyl isocyanate. The mixture of the derivatized higenamine enantiomers was applied to a reverse phase C_{18} column (10 μm), and eluted with 8% CH_3CN/0.05M TCA-0.05M NaH_2PO_4 (pH 2.8). *R*-(+)-higenamine-1-phenylethylurea could be separated from *S*-(-)-higenamine-1-phenylethylurea with the retention time of 14 min for the former and 20 min for the latter. However, quantitatively consistent data could not be obtained with the above method possibly because the derivatization did not proceed quantitatively.

The direct separation of the two enantiomers could be achieved with the employment of one of the commercially available chiral stationary phase, CHIREX-3020G-EO which *S-tert*-leucine and *R*-1-(α-naphthyl)-ethylamine have urea linkage to silica. The procedure was reproducible and sufficiently sensitive to allow for the detection of the individual enantiomers. Eluting with the mobile phase of hexane:

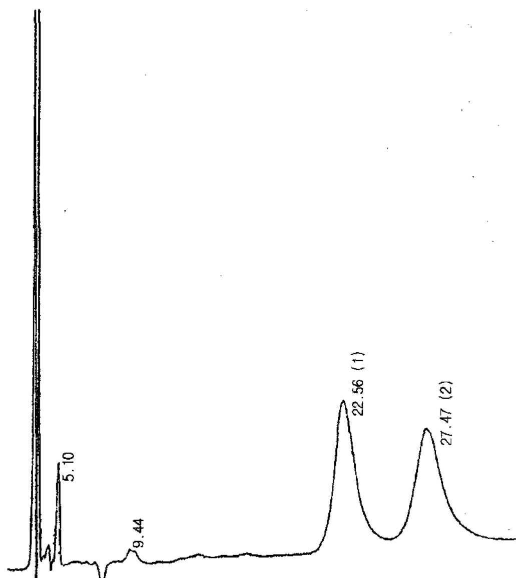


Fig. 1. A representative HPLC chromatogram of higenamine (racemic mixtures) Column:CHIREX 3020-G-EO Mobile phase:Hexane:Dichloroethane:TFA/EtOH (1/20) (53:35:12) Flow rate:0.8 ml/min. Detection: 284 nm (aufs:0.002) (1):*R*-(+)-higenamine, (2):*S*-(-)-higenamine.

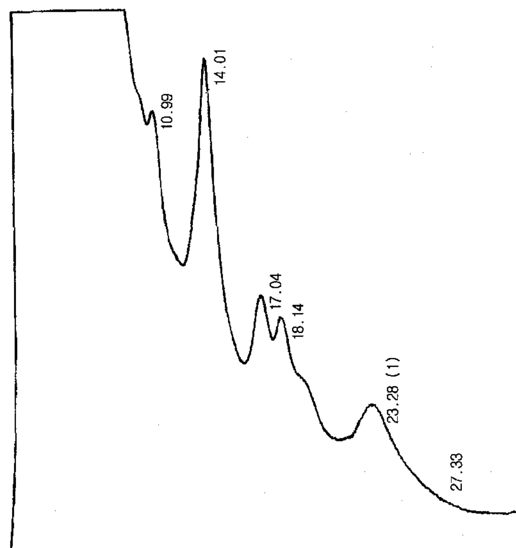


Fig. 2. A representative HPLC chromatogram of the extract of *Aconitum carmichaeli* after treatment with XAD-2 resin. (1) : *R*-(+)-higenamine. The analysis was performed at the condition described in Fig. 1.

dichloroethane:trifluoroacetic acid/EtOH (1/20) = 53:35:12 with a flow rate of 0.8 ml/min, the racemic mixture of higenamine provided two separate peaks at the retention times of 22.56 and 27.47 min as shown in Fig. 1. The enantiomer eluted at 22 min was identified as *R*-(+)-isomer and the other one eluted at 27 min was identified as *S*-(-)-isomer with the aid of pure authentic samples. *R*-(+)-higenamine

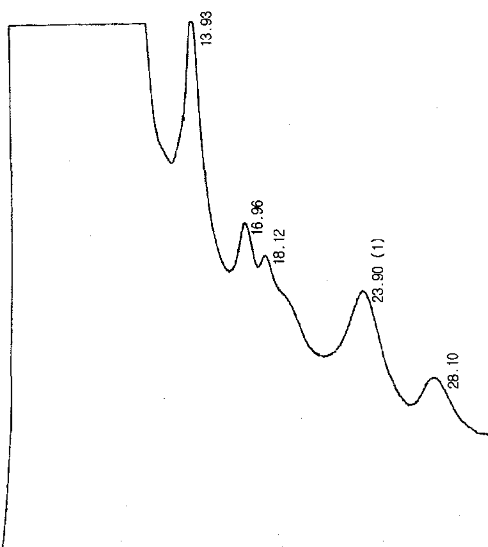


Fig. 3. A representative HPLC chromatogram of the extract of *Aconitum carmichaeli* after treatment with XAD-2 resin with the addition of standard racemic (*RS*)-higenamine (40.0 μg/ml), (1) : *R*-(+)-higenamine. The analysis was performed at the condition described in Fig. 1.

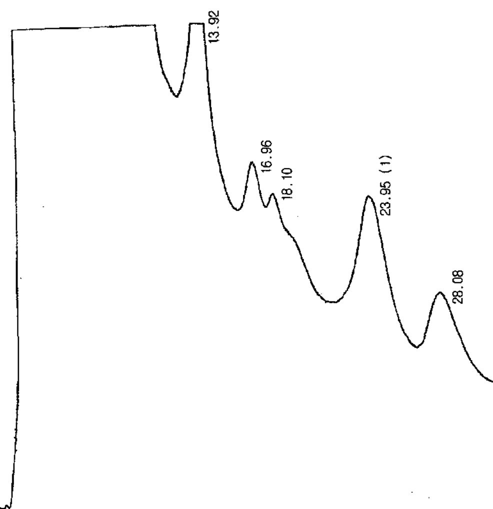


Fig. 4. A representative HPLC chromatogram of the extract of *Aconitum carmichaeli* after treatment with XAD-2 resin with the addition of standard racemic (*RS*)-higenamine (80.0 μg/ml), (1) : *R*-(+)-higenamine. The analysis was performed at the condition described in Fig. 1.

Table 1. Contents of *R*-(+)-higenamine in plant materials

Plant name	Higenamine (μg/g*)
Aconitum carmichaeli Debx. (root)	18.3
Aconitum carmichaeli Debx.(root processed)	12.2
Aconitum koreanum Rapaics (root)	19.1
Aconitum kusnezofii Rchb. (primary root)	8.2
Aconitum napiforme Lév. et Vent. (secondary root)	16.6
Nelumbo nucifera Gaertn. (embryo)	274.8
Processed Aconite root powder (*A. japonicum* Thunb.)	13.1

*g of crude plant

was reported to be separated from the embryo of *Nelumbo nucifera* and as expected, only the *R*-(+)-isomer was detected in this plant sample. In addition, *R*-(+)-isomer was also the only enantiomer detected in various *Aconitum* plant materials from which racemic higenamine was reported to be separated.

In the example chromatogram shown in Fig. 2, a peak appeared in the region of *R*-(+)-isomer (at 23.28 min) and no peak was detected in the region of *S*-(-)-enantiomer at around 27 min. With our findings, it was suspected that *R*-(+)-higenamine present in *Aconitum* sp. might be transformed to racemic mixtures during the extraction, separation and purification processes and was separated as optically inactive racemates.

The amounts of *R*-(+)-higenamine in plants were determined by the standard addition method. Since the extracts of plants contain various other components even after careful pre-treatments, the chromatograms with plant samples usually give many disturbing peaks other than the target peaks and thus interfere with the direct quantification of peak areas. An example of a calibration graph (Fig. 5) was obtained with the chromatograms Fig. 2 (*Aconitum carmichaeli* root), Fig. 3 and Fig. 4 (*Aconitum carmichaeli* root with the addition of racemic higenamine). The amount of *R*-(+)-higenamine was read as μg in 1 ml of injected solution and this refers to μg of higenamine in 1g of crude plant.

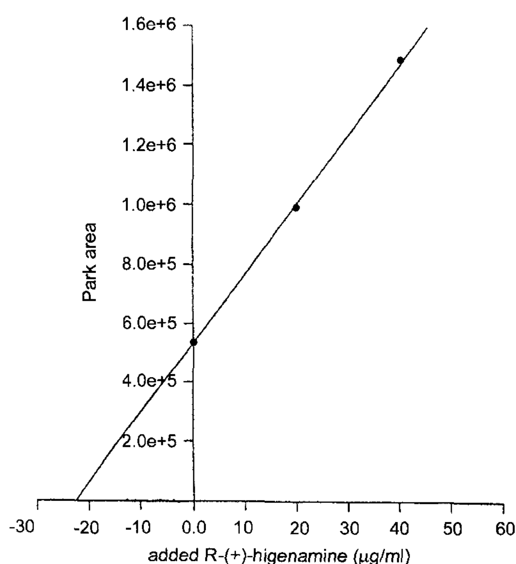


Fig. 5. A representative calibration curve for *R*-(+)-higenamne contents in *Aconitum carmichaeli* by standard addition method.

The results are summarized in Table 1. Roots of various *Aconitum* preparations contained 8-19 μg/g of *R*-(+)-higenamine while the embryo of *Nelumbo nucifera* had ten times more *R*-(+)-higenamine. The present data fairly well coincide with the data in which higenamine was analyzed as total (*R*- and *S*-) higenamine with non-chiral HPLC or GC/MSD (Chung, *et al.*, 1997) methods.

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